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Increases in muscle Ca^{2+} mediate changes in acetylcholinesterase and acetylcholine receptors caused by muscle contraction

(collagen-tailed acetylcholinesterase/calcium ionophore/cyclic nucleotides/muscle depolarization)

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ABSTRACT **The synthesis of acetylcholinesterase (AcChoE; acetylcholine acetylhydrolase, EC 3.1.1.7) and of acetylcholine receptors (AcChoR) by cultured rat muscle fibers is influenced strongly by the level of muscle contractile activity. If fibers are grown in the presence of tetrodotoxin (TTX) to block spontaneous contraction, the total amount of AcChoE decreases markedly, as does the percentage of AcChoE assembled as the collagen-tailed presumed synaptic form of the enzyme. Under these conditions, however, the number of AcChoR increases. We demonstrate here that each effect of TTX can be prevented by treating the muscle cells with the calcium ionophore A23187. Thus, cells treated with A23187 and TTX have 30- to 40-fold higher levels of collagen-tailed AcChoE and lower levels of AcChoR by a factor of 4–5 than do cells grown in TTX alone. These results suggest that an increase in muscle cytoplasmic Ca^{2+} mediates the known effects of muscle contraction on these cholinergic macromolecules.**

The junctional region of adult vertebrate muscle (1) is characterized by high concentrations of acetylcholine receptors (AcChoR) and acetylcholinesterase (AcChoE; acetylcholine acetylhydrolase, EC 3.1.1.7). A wide variety of studies, designed to explore the basis for the formation and maintenance of these junctional specializations, have suggested that three separable events are involved both during development and in adults: the clustering of junctional AcChoR, the suppression of extrajunctional AcChoR levels, and the insertion of synaptic AcChoE (2–4). These studies have also demonstrated repeatedly that although each event is regulated by some aspect of motor nerve–muscle interaction, the types of regulation may vary among the individual events. For instance, clustering of AcChoR is independent of synaptic transmission, appearing, instead, to be dictated at first by molecules of neuronal origin and ultimately by a component of the muscle's basal lamina (5–10). On the other hand, the levels of extrajunctional AcChoR and synaptic AcChoE seem to be strongly influenced by synaptic activity (10–14).

It would be of great interest to determine how synaptic activity influences levels of these molecules because neuronal activity is, in general, a potent modulator of nervous system function. In fact, it appears that muscle contraction, a normal consequence of synaptic activation at the neuromuscular junction, is of central importance in regulating AcChoR numbers because direct electrical stimulation of adult muscle and spontaneous contraction of cultured myotubes are both associated with decreased numbers of AcChoR (11, 12, 15, 16). This occurs as a result of decreased AcChoR synthesis (17). Of course, contraction itself is a complex process normally associated with muscle depolarization and with at least transient increases of Na^+ and Ca^{2+} (18) and cyclic GMP (19). Altered muscle AcChoR levels have been attributed alternatively to increased cyclic GMP (20) and to modifications in Ca^{2+} distribution (21–24).

Muscle contraction has a prominent role in control of AcChoE levels as well. Direct electrical stimulation of embryonic and adult muscle causes synaptic AcChoE appearance even in the absence of synaptic transmission (10, 14). The requirement for muscle activity is probably involved, at least in part, with a greatly enhanced assembly of the A12 form of the enzyme (10, 13). This enzyme species is composed of three sets of four catalytic subunits disulfide bonded to a collagen-like tail and is thought to be the one present in muscle basal lamina (25–27). Numerous investigators have shown that a variety of types of muscle fibers grown in neuron-free primary cell culture and permitted to undergo spontaneous contraction are able to synthesize several forms of AcChoE including the A12 (28–31). However, if contraction of rat muscle fibers, for example, is blocked by growing the fibers in the presence of tetrodotoxin (TTX), both the total amount of AcChoE and the percentage of AcChoE assembled as the A12 form are decreased severalfold (32–34). In this paper, we present data that suggest an increase in muscle cell Ca^{2+} in the absence of contraction is able to stimulate cultured muscle cells to synthesize greatly increased amounts of A12 AcChoE. We also show that similar conditions cause an impressive decrease in the level of cell-surface AcChoR.

METHODS

Cell Culture. Myoblasts were dissociated from 20- to 21-day-old rat embryos following standard procedures (35). They were grown on collagen-coated plastic dishes in a culture medium consisting of 80% Dulbecco's modified Eagle's medium (DME medium) and 20% fetal calf serum (HyClone, Logan, UT), supplemented with 600 mg% glucose and antibiotics. After 2 days, the medium was changed to 88% DME medium/10% horse serum (Kansas City Biologicals, Lenexa, KS)/2% chicken embryo extract, again supplemented with glucose and antibiotics. Cells started to contract spontaneously 5–7 days after being plated; at this time, drug treatment normally began and continued for 48 hr. Cultures were not routinely treated with mitotic inhibitors because they remained more stably attached to the culture surface when fibroblasts were present, especially when undergoing frequent contraction. This made no difference in the distribution of AcChoE forms.

For some experiments, as indicated below, the cells were grown in a defined medium modified from that of Bottenstein and Sato (36) and consisting of F-12/DME medium (1:1; Irvine Scientific), bovine serum albumin (100 μg/ml; Collaborative Research), conalbumin (300 μg/ml; Sigma), progesterone (20 nM; Sigma), putrescine (100 μM; Sigma), insulin

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Abbreviations: AcChoE, acetylcholinesterase; AcChoR, acetylcholine receptor(s); TTX, tetrodotoxin.

(5 μg/ml; Collaborative Research), selenium (30 nM; Sigma), with additional sodium bicarbonate (18 mM) and glucose (600 mg%).

Drugs used included TTX (Sigma), veratridine (Sigma), A23187 (Calbiochem), ionomycin (Calbiochem), dibutyryl cyclic AMP (Sigma), forskolin (Calbiochem), dibutyryl cyclic GMP (Sigma), and sodium nitroprusside (Sigma).

AcChoE Extraction and Sucrose Density Gradients. To measure cellular AcChoE forms, cultures were rinsed twice with DME medium and once with ice-cold phosphate-buffered saline. Cells were scraped off the dishes and homogenized in a buffer containing 1 M NaCl, 0.5% Triton X-100, and antiproteases as described (31, 37). The homogenate was centrifuged in a Microfuge for 10 min, and the supernatant was layered on 5–20% sucrose gradients (made up in 1 M NaCl/0.5% Triton X-100; ref. 25). Supernatants were assayed for activity by using a radiometric procedure modified (31) from that of Johnson and Russell (38) and Rand and Johnson (39) with [^{3}H]acetylcholine (Amersham) as the substrate. They were assayed for protein content by the procedure of Schaffner and Weissmann (40). Gradients were centrifuged for 18 hr at 40,000 rpm in a Beckman SW40 rotor, fractionated, and AcChoE activity was assayed following the procedure of Ellman *et al.* (41).

Secreted AcChoE Forms. For analysis of secreted enzyme forms, cells were grown for various periods in the chemically defined medium described above. The medium was collected, and secreted AcChoE was concentrated ≈5-fold by using an Amicon CF-50A ultrafiltration filter. AcChoE activity was assayed before and after this procedure, using the radiometric procedure described above; >90% of enzyme activity was retained by these filters. The concentrated supernatants were centrifuged and analyzed as described above.

Surface AcChoR Levels. Sister cultures to the ones used for AcChoE analyses were labeled with 5 nM ^{125}I-labeled α-bungarotoxin (Amersham) for 75 min at 37°C. Cultures were incubated for 30 min with 1% Triton X-100/phosphate-buffered saline to extract membrane AcChoR. Triton-soluble extracts were counted in a Packard γ counter.

RESULTS

A12 AcChoE Synthesis Is Enhanced by Calcium Ionophore Treatment. As reported previously (32–34), rat myotubes start to contract spontaneously 4–7 days after plating and then synthesize three primary AcChoE forms, all present as significant percentages of the total: the globular forms, G1 and G4, and the collagen-tailed asymmetric form, A12. There were also variable amounts of another asymmetric form, A8. If contraction was blocked by growing the fibers in the presence of the sodium channel blocker TTX, both the total amount of AcChoE and the percentage of AcChoE assembled as the A12 form were decreased severalfold; as a result, the G1 form became much more dominant (Table 1). On the other hand, if spontaneous contraction was blocked by treating cells with veratridine, which opens sodium channels, levels of AcChoE were substantially increased (relative to those of cells grown in TTX), and the percentage of A12 AcChoE was similar to that in untreated cultures (Table 2; see also ref. 42). This suggests that the mechanical component of muscle contraction is unimportant; furthermore, muscle action potentials also appear not to be involved in AcChoE induction because they are also blocked by veratridine treatment. Rather, cellular modifications associated with muscle depolarization, sodium influx, or with secondary calcium influx—all of which are caused by both contraction and veratridine—must be essential. Of course, changes in cyclic nucleotides or in other cellular metabolites could still act as mediators.

To determine the basis for the pronounced enhancement of AcChoE activity, we studied the levels and distributions of AcChoE forms in cultures incubated with a variety of compounds in addition to TTX. We attempted, in this way, to identify treatments that, like veratridine, would mimic the effects of contraction in stimulating the activity of the A12 form, but in the absence of any actual contraction.

A variety of treatments had little effect on muscle AcChoE (Table 2). Increasing extracellular K^+ to depolarize muscle fibers in the presence of TTX was ineffective; in fact, increased levels of K^+ alone blocked contraction and were as effective as TTX in suppressing total activity of the A12 enzyme form. Thus, depolarization itself is not a substitute for contraction. Presumably, then, the important cue is a consequence of the enhanced Na^+ influx occurring during both frequent muscle contraction and veratridine treatment and would only be transient during maintained depolarization because of Na^+ channel inactivation (see *Discussion*).

Alterations in cyclic nucleotide levels are well known mediators of a variety of cellular processes, and changes of cyclic GMP have been measured in muscle after direct or indirect stimulation (19). Thus, cultures grown in TTX were supplemented with dibutyryl cyclic AMP or forskolin (both of which increase cellular cyclic AMP levels; see ref. 43), dibutyryl cyclic GMP or sodium nitroprusside (which increase cellular cyclic GMP; see ref. 44). In all cases, the percentage of AcChoE assembled as the A12 form remained <5%, and total enzyme activity was not altered (Table 1).

Another important change known to be associated with muscle contraction is a transient increase in intracellular Ca^{2+} levels. To examine the effects of increased muscle Ca^{2+}, we treated cells with TTX and various concentrations of the calcium ionophore A23187. When cells were treated with 0.5–1.0 μM A23187, there was a dramatic shift in the distribution of forms so that the A12 and G4 forms became more dominant, seemingly at the expense of the G1 form

Table 1. Effect of A23187 on AcChoE and AcChoR levels

Treatment	AcChoE specific activity, relative units	AcChoE form, %				AcChoR, cpm per dish
		A12	A8	G4	G1	
Control	841	20.8 ± 1.9 (175)	9.0 ± 1.2 (75.9)	34.5 ± 2.8 (290)	35.7 ± 1.1 (300)	56,661 ± 5,795
TTX (10 μM)	100	6.3 ± 1.6 (6.3)	2.4 ± 0.7 (2.4)	39.2 ± 2.0 (39.2)	52.2 ± 2.7 (52.2)	90,930 ± 15,786
TTX/1 μM A23187	769	26.8 ± 3.2 (206)	6.9 ± 1.9 (53.4)	51.1 ± 4.2 (393)	15.3 ± 2.0 (118)	21,655 ± 2,647

AcChoE specific activity was calculated from AcChoE activity and protein values of cell extracts. All numbers were normalized to that for TTX, which was given a value of 100 units. Forms are shown as means ± SEM for a minimum of 10 separate determinations. Numbers in parentheses represent total relative amounts of each enzyme form (i.e., percent × total specific activity). AcChoR levels are given as cpm per dish ± SEM for 5 separate experiments. Each experiment represents an average of 2.4 dishes.

Table 2. Distribution of AcChoE forms after various treatments

Treatment	AcChoE specific activity, relative units	AcChoE form, %			
		A12	A8	G4	G1
Veratridine (2 μg/ml)	240	25.9	7.0	32.9	34.3
TTX/5 μM ionomycin	305	35.9	6.7	42.4	15.2
KCl (20 mM)	86	1.2	1.4	42.5	54.7
TTX/20 mM KCl	91	3.2	0.8	46.6	49.4
TTX/250 μM dibutyryl cyclic AMP	105	1.4	2.9	52.1	42.3
TTX/10 μM forskolin	75	0.8	0	49.9	49.4
TTX/250 μM dibutyryl cyclic GMP	105	4.0	0	37.4	58.6
TTX/100 μM sodium nitroprusside	125	1.0	0	29.0	70.0

AcChoE specific activity was calculated as described in the legend to Table 1. At least two determinations were made for each treatment.

(Table 1; Fig. 1). In fact, ionophore-treated cultures occasionally had substantially more of the A12 and A8 forms than did control cultures. Muscle cells in these cultures also had markedly more total AcChoE activity than did those grown in TTX alone. Overall, there was more than a 30-fold difference in the total specific activity of A12 AcChoE between ionophore- and TTX-treated cultures. Treatment with another Ca^{2+} ionophore, ionomycin, in the presence of TTX, had generally similar effects to those of A23187 (Table 2).

Time Course of Ionophore Effects. Before substantial changes in A12 activity could be detected, cells had to be treated with ionophore for 24–48 hr. Enhanced enzyme activity was maintained for at least 96 hr of ionophore treatment. If cells were examined after a short time interval (6 hr), there was little, if any, enhancement of the percentage of AcChoE assembled as the A12 form. As shown in Fig. 2, after 6 hr of A23187 treatment, A12 and G4 levels were similar to those in cells grown in TTX alone, but levels of the G1 form were significantly smaller. Total cellular levels of AcChoE at this time were, in fact, only 53.1% ± 6.5% (mean ± SEM) of those grown in TTX alone.

Since enhanced intracellular Ca^{2+} is known to mediate secretion from many cell types (45), we investigated secretion of AcChoE after ionophore addition. Muscle cells were grown in a chemically defined medium, which was collected at various times and analyzed for enzyme forms by sucrose density gradient centrifugation. Brief periods of treatment with defined medium alone had little, if any, effect on total AcChoE activity or distribution of enzyme forms. Total AcChoE activity found in the medium 6 hr after the beginning of ionophore addition was 202% ± 16% of that found in cells maintained in TTX alone. Surprisingly, although G4 was the predominant secreted form when cells were grown in TTX alone, G1 was the form whose secretion was substantially increased by A23187 treatment. This secretion accounts quantitatively for the observed difference in the cellular levels of this form. The enhanced secretion persisted for at least 24 hr.

Ionophore Treatment Causes a Substantial Decrease in Surface AcChoR Levels. Since ionophore treatment was so effective in reproducing the influences of normal contractile activity on AcChoE levels and forms, we also determined its effects on surface AcChoR numbers in these cells. Sister cultures to the ones used for AcChoE measurements were labeled with ^{125}I-labeled α-bungarotoxin and were detergent-extracted to solubilize membrane AcChoR. Control cultures

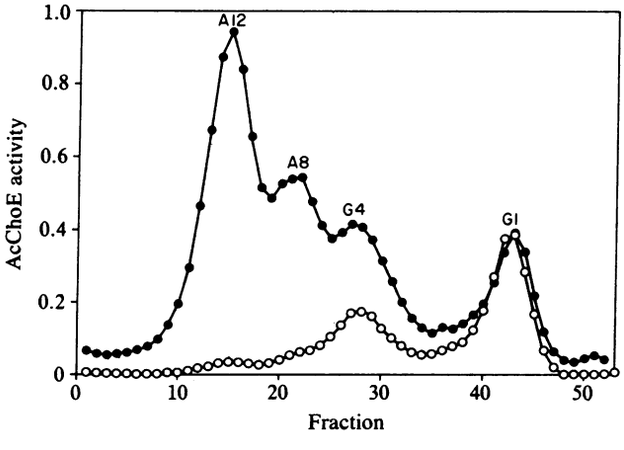

FIG. 1. Sucrose density gradients of TTX- and A23187-treated cultures. Cells were grown for 48 hr in either TTX alone (○) or in TTX/1 μM A23187 (●). AcChoE was extracted from identical numbers of the two types of cultures and was fractionated into its four major forms on sucrose density gradients; the major enzyme forms are labeled. Ionophore treatment causes large increases in the total activity of the A12, A8, and G4 forms.

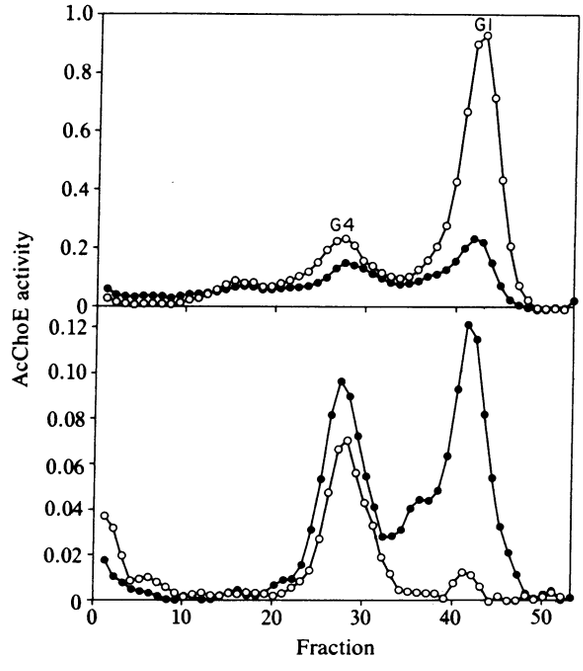

FIG. 2. Cellular and secreted AcChoE forms 6 hr after A23187 treatment. (*Upper*) Cellular forms. (*Lower*) Secreted forms. Identical numbers of cultures were grown in a chemically defined medium with either TTX (○) or TTX/A23187 (●). Ionophore-treated cells have lower amounts of the G1 form at this early time because they preferentially secrete large quantities of it.

had somewhat fewer AcChoR than did those treated with TTX (Table 1), as reported previously (e.g., see refs. 15 and 16). Cultures treated in the presence of TTX with the same concentrations of A23187 (0.5–1.0 μM) which were effective in enhancing AcChoE levels, however, had many fewer AcChoR than did the other types of cultures (Table 1). This suppression was probably due to decreased synthesis, because the AcChoR turnover rate was actually somewhat slowed (unpublished data).

DISCUSSION

The activity dependence of synaptic AcChoE appearance has been recognized for some time and has been shown to depend, at least in part, on a markedly enhanced stimulation of the A12 enzyme form, which has been observed both *in vivo* and *in vitro*. Data presented in this paper confirm previous results (32, 34), revealing that blocking spontaneous contraction of cultured rat muscle fibers with TTX has marked effects on AcChoE levels and forms. Neither depolarization with a high concentration of K^+ nor manipulation of cyclic nucleotide levels overcame these effects. On the other hand, addition of veratridine alone or of either of two calcium ionophores in the presence of TTX increased total AcChoE activity and the percentage of the enzyme synthesized as the A12 form. In general, therefore, these noncontracting muscle fibers—particularly those treated with the Ca^{2+} ionophores—were similar to those undergoing the normal degree of spontaneous contraction. Previously, De La Porte *et al.* (42) found veratridine, but not A23187, effective in stimulating A12 AcChoE synthesis. We are not able to account for the differences between their studies and ours, but we rely on our observation that both of the Ca^{2+} ionophores we used had generally similar effects. It could be that variations in culture procedures explain these differences, but we have found that ionophore treatment certainly could be carried out at any time during a several day period beginning with the normal onset of muscle contraction. Our current results are also different from those we obtained during our earlier studies of TTX-paralyzed chicken nerve–muscle cultures in which we found that dibutyryl cyclic GMP treatment promoted the appearance of synaptic AcChoE (10). Changes in A12 AcChoE synthesis seen then were much less dramatic, however, than those measured here.

We also found that identical A23187 treatment reproduces another effect known to be associated with muscle contractile activity: a decrease in the total number of cell-surface AcChoR. In this case, the decrease was much more significant than that produced by the normal amount of spontaneous contraction. It has been shown by several investigators that ionophore treatment produces alterations in the levels of a variety of muscle cell components, including oxidative and glycolytic enzymes (46–48). It may be, then, that an increase in muscle cell Ca^{2+} underlies the ability of muscle contraction to modulate amounts and forms of numerous muscle cell components. The different actions could well be mediated by different levels of intracellular Ca^{2+} and by separate Ca^{2+}-mediated processes (49).

There are three likely sources for increased muscle Ca^{2+} during action potentials and contraction—flux through membrane ion channels during acetylcholine action or membrane depolarization, release from the sarcoplasmic reticulum during contraction, and a putative Na^+/Ca^{2+} exchanger known to be present in other muscle and excitable cell types (50, 51). It may seem somewhat inconsistent with our suggested involvement of increased cell Ca^{2+} that veratridine, but not high K^+ concentration, was able to stimulate AcChoE activity because it is presumably depolarization that normally causes Ca^{2+} influx through plasma membrane ion channels and release of Ca^{2+} from the sarcoplasmic reticulum. However, it has been demonstrated that prolonged depolarization with high K^+ concentration causes inactivation of the sarcoplasmic reticulum Ca^{2+} release mechanism (52) and also of membrane Ca^{2+} channels (53, 54), so that depolarized cells would not undergo a continuous increase in Ca^{2+}. In addition, since Na^+ channels inactivate after prolonged depolarization, paralyzed cells would not undergo periodic increases in Na^+ either. Veratridine treatment, on the other hand, would increase intracellular Na^+ and might well increase intracellular Ca^{2+} via a Na^+/Ca^{2+} exchange.

A significant aspect of the present results is that increased Ca^{2+} shifts the distribution of AcChoE forms. Within hours of ionophore addition, there is a marked increase in secretion of the G1 form in spite of the fact that G4 is normally the major secreted form. A much slower effect is an enhancement of the activity of the A12 form, but both effects could well be related to similar changes in intracellular processing of the catalytic subunits. The time course of the increase in A12 suggests an influence on protein synthesis, rather than on enzyme activation or assembly, and our recent experiments make it likely that this is the case (unpublished data). A likely hypothesis is that after a certain period of normal contraction or after a direct increase in Ca^{2+}, there is dramatically enhanced synthesis of the collagen tail subunit of the A12 form, which then becomes disulfide bonded to sets of catalytic subunits in muscle Golgi apparatus (55).

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